## III

## THE SYNTHESIS OF GALLSTONES

THE problem of the mechanism of the formation of gallstones has claimed the attention of numerous investigators from the very beginning of the art of medicine. But in spite of all the work that has been done, there is still considerable difference of opinion as to the origin of these troublesome stone-like bodies which all too frequently are deposited in the gall bladder or ducts of both man and animals. In the course of this lecture it is proposed to give an account of some recent investigations carried out by Mr. Gray and myself on the synthesis of the most common types of human gallstones. It is hoped that the results of this work will throw light on an age-old problem and will be helpful in its ultimate solution.

The modern view of the mechanism of gallstone formation is the direct outgrowth of recent developments in the science of colloid chemistry. Although the colloidal theory of gallstone formation is largely the product of recent investigations, it should be recalled that what is now termed colloidal behavior was recognized as important in producing concretions by Hippocrates, the Father of Medicine, in the fourth century B. C. and by Galen in the second century of our era. These renowned physicians of ancient Greece attributed the abnormal deposits to an accumulation of mucus which clung to the organ and served as a nucleus for the stone which subsequently formed. The first experimental evidence of the rôle which colloids may play in concrement formation

was obtained in 1684 by A. von Heyde who dissolved out the crystalline material from a urinary calculus and observed a residual framework. This organic framework was recognized quite clearly by Meckel von Hemsbach as evidenced by the following quotation from his book, *Mikrogeologie,* published in 1856. "Two basic factors underlie the formation of every true gall or urinary stone; first, the presence of an organic substance, mucus, in which there may be depositions of salts; second, a suitable urinary or gall fluid to serve as the mother liquor for these sediments. The decomposable organic substance, mucus, is unquestionably necessary, because urinary salts and gall substances of themselves can yield only crystalline, pulverulent or granular precipitates and never larger pieces. Stones are formed only when an organic binder is carried down too."

While the presence of a colloidal organic binding material resulting from an inflammatory process has been definitely established as essential for the formation of certain types of concretions,[1] it was demonstrated almost a quarter of a century ago by Aschoff and Bacmeister[2] and by Schade[3] that both gall and urinary calculi may form under suitable conditions without inflammation as a result of infection.

[1] Pfeiffer: *5th Cong. Int. Med.,* Wiesbaden (1886); Posner: *Arch. klin. Med.,* **5,** (1885); **16** (1889); Naunyn: *Klinik der Cholelithiasis,* Leipzig (1892); Gilbert and Dominici: *Compt. rend. soc. biol.,* **28,** 1033 (1893); Moritz: *14th Cong. Int. Med.,* Wiesbaden (1896); Gilbert: *Arch. gén. de Méd.,* **2,** 257 (1898); Gilbert and Fournier: *Presse Méd.,* **7,** 259 (1898); Mignot: *Arch. gén. de Med.,* **129,** 263 (1898); Schrieber: *Virchow's Archiv,* **153,** 147 (1898); Cushing: *Bull. Johns Hopkins Hosp.,* **10,** 166 (1899).

[2] *Cholelithiasis* (1909); *Lectures on Pathology,* 206 (1924); Kleinschmidt: *Die Harnsteine* (1911).

[3] *Münch. med. Wochenschr.,* Nos. 1 and 2 (1909); *Kolloid-Z.,* **4,** 175, 261 (1909); *Kolloid-Beihefte,* **1,** 371 (1910); Alexander's *Colloid Chemistry,* **2,** 801 (1928); cf., also, Boysen: *Über die Struktür und Pathogenese der Gallensteine* (1909).

## COMPOSITION OF THE BILE

Since the crystalline constituents of gallstones are derived from the bile, consideration will be given first of all to the nature and composition of this secretion.

The bile or gall is a viscid, yellowish or greenish liquid consisting of solids dissolved or dispersed in water. Since the secretion is concentrated in the gall bladder, gall bladder bile always contains more of the solid constituents than common duct bile, the concentration increasing with the length of time of storage. Rous and McMaster[1] showed that in certain cases the concentration may be increased as much as ten fold in twenty-four hours. The second and third columns of the following table give the average composition of normal human common duct and gall bladder bile, respectively, from a number of sources as reported by Rosenbloom.[2] In the last column of the table is given the manner in which the several solid constituents are held in the bile fluid.

| Constituents | Percentage composition | | State |
|---|---|---|---|
| | common duct | gall bladder | |
| Water | 98 | 86 | ——— |
| Inorganic salts NaCl, $CaCl_2$, $CuCl_2$, etc. | 0.7 | 0.8 | In molecular solution |
| Sodium glycocholate and taurocholate | 0.9 | 9.1 | Partly in molecular solution but chiefly as colloidal electrolytes dispersed like the soaps |
| Mucin and pigments | 0.5 | 2.6 | In colloidal solution |
| Lecithin and fat | 0.3 | 0.8 | Emulsified by the cholates |
| Cholesterol | 0.12 | 0.48 | Peptized by the alkali cholates; dissolved in the emulsified fat; and collected around the droplets. |

[1] *J. Exptl. Med.*, **34**, 47 (1921).

[2] *J. Biol. Chem.*, **14**, 241 (1913).

The chief inorganic salts in the bile are the chlorides of sodium, potassium, and calcium but traces of iron, copper, and zinc salts may be present.

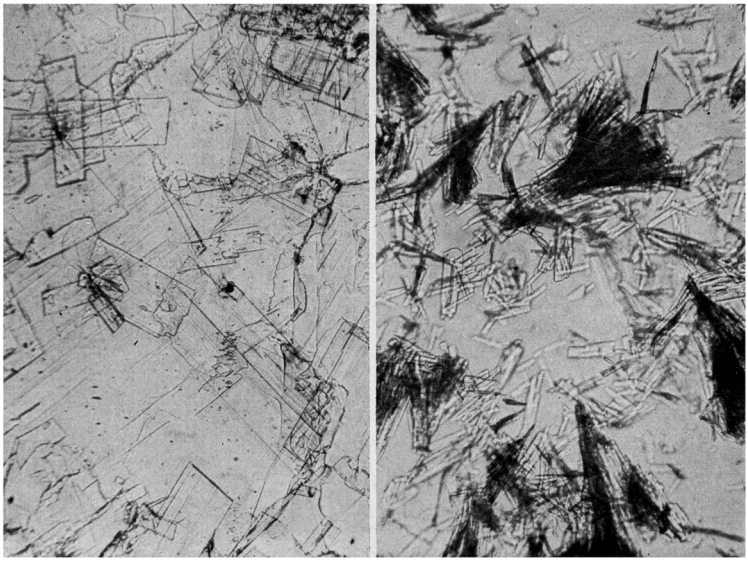
The bile salts, sodium glycocholate and taurocholate, are the most important constituents of the bile. Like the soaps they are exceptionally good emulsifying agents and their rôle in the process of digestion is to emulsify the fats in the foods. They also retain the fatty constituents of the bile in the form of an emulsion. Their extremely bitter taste is responsible for the well known phrase, "bitter as gall."

The biliary mucin secreted by the gall bladder wall is a glucoproteid like that in the saliva. Its function may be to lubricate the bladder bile, which becomes thick and tenacious on standing and it may serve to facilitate the passage of gallstones.

The coloring matter of the bile is chiefly bilirubin, which is reddish brown, and biliverdin, an oxidation product of bilirubin, which is green. These compounds are insoluble in water but dissolve in the alkaline bile probably forming colloidal electrolytes the colloidal anions of which are colored. The calcium salts of these pigments are insoluble and are the chief constituents of the so-called "pure" calcium-pigment gallstones which are sometimes encountered.

Cholesterol was first found in gallstones and for this reason was given the name solid bile (from the Greek *chole,* bile; *stereos,* solid). It is the chief constituent of nearly all biliary concretions and is present in greater or less amount in all of them. In the pure state it is a white, light, flaky, crystalline solid insoluble in water but fairly soluble in both alcohol and fats. The form of the crystals deposited from the saturated solutions in the respective solvents is shown in Fig. 1, (A) and (B).

It is commonly stated that the cholesterol is held in the



A B

FIGURE 1. Cholesterol crystals deposited from (A) alcohol; (B) fat. (x70).

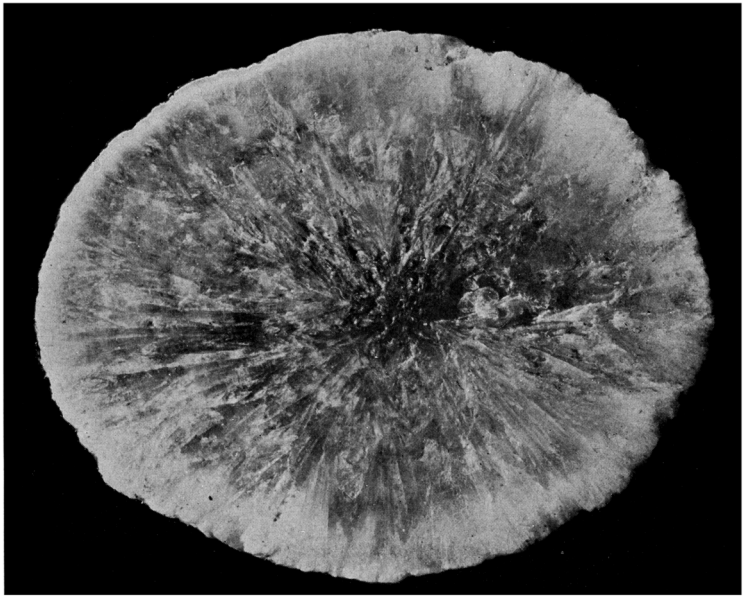


FIGURE 2. Cross-section of pure cholesterol gallstone. (x6).

dispersed state in the bile by the peptizing action of the alkali cholates. This statement we have found to be only partially correct. Fat-free cholesterol was suspended in an 8 per cent solution of sodium glycocholate and digested for several days at 37°C, the body temperature. The amount taken up corresponds to but 0.2 gram in 100 cc of solution or one part of cholesterol to forty parts of bile salt. In contrast to the relatively low peptizing action of the cholates is the marked solubility of cholesterol in fats. Thus at body temperature 100 cc of olive oil dissolves 6 grams of cholesterol. Since the bile may contain one per cent or more of fatty material it is apparent that one-third to one-half the cholesterol in normal bile is dissolved in the emulsified fat. Moreover, as we shall see, any excess cholesterol tends to concentrate around the fat droplets.

### GALLSTONES FORMED WITHOUT INFECTION

Stones formed in the gall bladder without the coexistence of inflammation resulting from infection are usually designated as "pure" stones, as for example, "pure cholesterol stones" or "pure calcium pigment stones." The cholesterol stones, which are much the more common, will be taken as the example of this type. Since such stones always occur singly, Meckel von Hemsbach[1] called them "cholesterol solitaires." They consist largely of cholesterol, although fat and small amounts of alkali and calcium cholates, and bile pigments, are usually present. Fig. 2 shows a cross section of a stone which was nearly pure cholesterol. The specimen was white throughout hence was free from bile pigments. A portion of the specimen was found to be almost completely soluble in ether, indicating that it was largely cholesterol and fat.

[1] *Mikrogeologie* (1856).

The incidence of pure cholesterol stones is believed by Aschoff[1] to result from a disturbed metabolism which gives an abnormally high content of cholesterol in the blood and subsequently in the bile. According to Dostel and Andrews[2] a survey of the experimental evidence does not support this view; on the contrary, it shows the absence of any connection between the cholesterol content of blood and bile. Quite recently, however, Wilkie and Doubilet[3] at McGill have made some striking observations which support Aschoff's theory. Thus it was shown that in normal animals with the cystic duct tied, cholesterol passes from the blood through the mucosa of the gall bladder into the bile provided the cholesterol concentration of the bile is lower than that of the blood, whereas cholesterol passes from the bile through the gall bladder into the blood stream provided the cholesterol concentration of the bile is higher than that of the blood. Moreover, the amount as well as the direction of passage of the cholesterol appears to depend on the blood-bile cholesterol ratio.

In contrast to Aschoff's theory, Naunyn[4] attributes the presence of excess cholesterol which may lead to stone formation in the gall bladder to the disintegration of gall bladder epithelium or to the direct secretion of cholesterol by the gall bladder mucosa. This view has been pretty largely disproved and has been abandoned by most pathologists. Recently, however, it has been revived by Elman and Graham[5] to account for the presence of cholesterol crystals in the walls of the gall bladder. While this pathological condition called cholesterosis or "strawberry" gall bladder is

[1] *Lectures on Pathology,* 206 (1924).
[2] *Arch. Surgery,* **26,** 258 (1933).
[3] *Arch. Surgery,* **26,** 110 (1933).
[4] *Klinik der Cholelithiasis* (1892).
[5] *Arch. Surgery,* **24,** 14 (1932).

well known, it does not follow that the crystals deposited in the gall bladder mucosa are secreted by the mucosa.

On one point everybody is agreed: whatever the source of the excess cholesterol, a condition must arise which causes precipitation of the compound or no stone can form. During stasis, Aschoff visualizes the precipitation from a hypercholestolated bile about some nucleus, ultimately leading to the formation of a gallstone. While this step is necessary, pathologists have been slow to recognize that precipitation is in itself altogether inadequate to account for the binding together of the crystalline particles into a concretion. Schade[1] realized the necessity of accounting for the collecting together of the precipitated cholesterol into a coherent mass and proposes the following mechanism:

> Bile always contains, in addition to cholesterol, small quantities of fat dissolved in its cholate. Now in stasis of the bile, as the experience of the surgeon and the pathologist proves, the concentration of the cholate is gradually diminished by autolysis and resorption until finally a water-clear, almost cholate-free fluid is left; but the cholesterol remains in undiminished quantity and is ultimately in excess. The increasing impoverishment of the bile in cholate content, compels small quantities of cholesterol to separate out from time to time. But owing to the presence of fat it is guttulate separation which occurs, and since in such simple stasis, foreign substances are lacking, there is nothing to prevent the aggregation of the droplets.

While Schade's proposed mechanism possesses elements of value it is based on certain misconceptions which render it inadequate. In the first place, it is taken for granted that the alkali cholates can hold large amounts of cholesterol in the dispersed state; but, as we have seen, it requires in the neighborhood of forty parts of cholate to disperse one part of cholesterol. In the next place it is assumed that during stasis the cholates gradually disappear allowing the cholesterol to precipitate. But Rous and McMaster[2] and more

[1] Alexander's *Colloid Chemistry*, **2**, 801 (1928).

[2] *J. Exptl. Med.*, **34**, 47 (1921).

recently Andrews, Dostal, Goff, and Hrdina[1] have demonstrated conclusively that stasis alone does not necessarily alter the bile salt-cholesterol ratio in the gall bladder. The introduction of some other factor such as bacterial infection or chemical irritation, say by pancreatic juice, is necessary for resorption of the bile salts to take place. But inflammation from infection, or, indeed, anything more than a relatively mild chemical inflammation, is not essential for the formation of pure cholesterol solitaires since gall bladders in which they are found usually show no signs of inflammation and the pathological results of infection are absent. Moreover, in the presence of infection, stones of a distinctly different type are formed.

Finally, emphasis is laid on the importance of the presence of excess fat; but, as we have seen, cholesterol is quite soluble in fat and an excess of the latter would tend to prevent rather than favor the formation of gallstones.

The following experiments appear to throw considerable light on the mechanism by which precipitated cholesterol may be collected into a unified coherent mass.

1. A drop of olive oil was shaken with 10 cc of a 6 per cent solution of sodium glycocholate. A stable emulsion was formed in which the droplets vary in size from extremely minute to fairly coarse. Fig. 3 shows the appearance of a portion of this emulsion magnified seventy times. The emulsion is quite stable but tends to "cream" on standing giving an upper layer that is relatively richer in fat than the lower layer.

2. A drop or two of olive oil saturated with cholesterol at 50° was added to 10 cc of the sodium glycocholate solution at 25° and the mixture shaken vigorously. An emulsion was again formed but this time there was present an

[1] *Ann. Surgery*, **96**, 615 (1932).

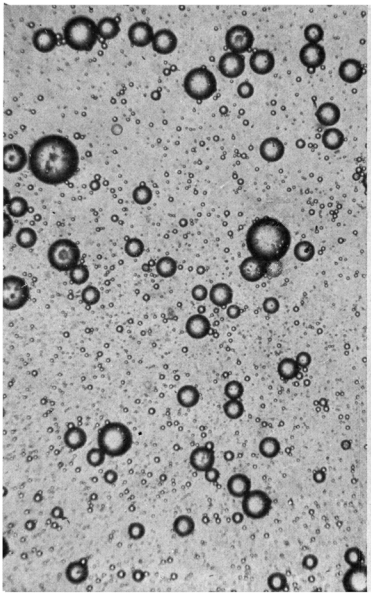


FIGURE 3. Fat emulsified by sodium glycocholate. (x70).

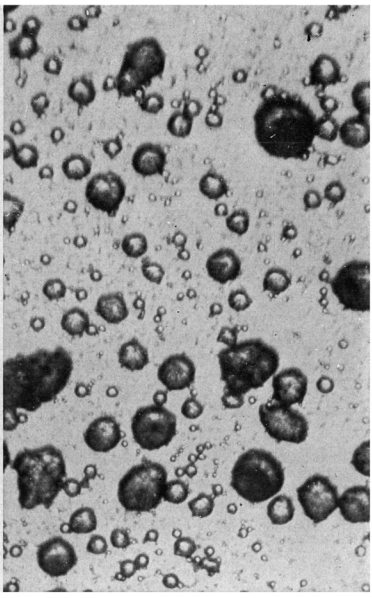


FIGURE 4. Emulsified fat droplets surrounded by cholesterol. (x70).

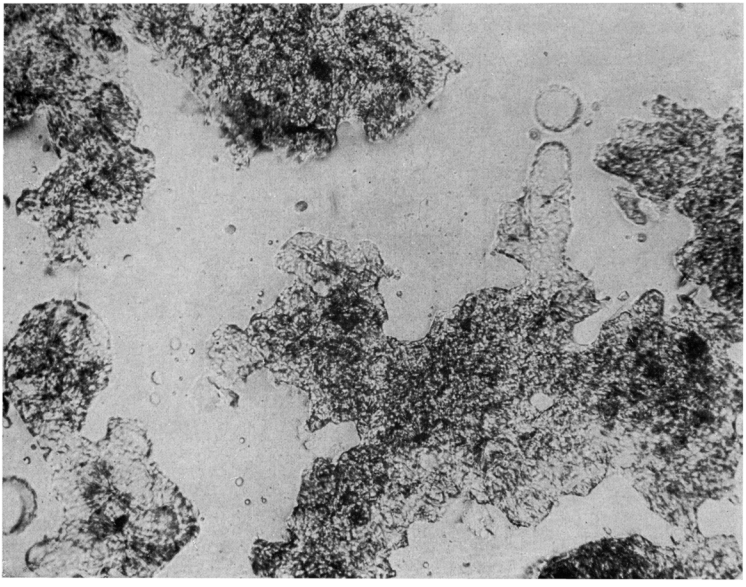


FIGURE 5A. Growth of cholesterol crystals in a cholesterol-fat clump. Original clump. (x70).

amount of free cholesterol in excess of the amount required to saturate the fat at 25°. A small portion of this excess cholesterol is peptized by the alkali cholate and the remainder concentrates on the surface of the droplets of emulsified fat. This is shown very clearly in Fig. 4. It is interesting to note that under these conditions the fat droplets have an "armor plate" of cholesterol as evidenced by the fuzzy appearance of the edges of most of the fat droplets and by the distortion in shape of many of them. It is apparent that cholesterol has a marked tendency to collect at an oil-water interface. For this reason, in the bile any excess cholesterol over and above that dissolved in the bile fat and peptized by the alkali cholates, concentrates at the surface of the droplets. It thus appears that the chief rôle of the bile cholates in holding relatively large amounts of cholesterol in colloidal dispersion is as an emulsifying agent for the fats, thereby furnishing a relatively large surface on which minute particles of cholesterol are adsorbed and prevented from settling out.

3. Like most emulsions, those considered in the preceding paragraph "cream" on standing, that is, the upper portion becomes relatively richer in fat. This process is accompanied by some coalescence, especially of the larger drops, into still larger units. When the droplets are coated with a film of cholesterol there is a marked tendency to form clumps of droplets. Thus after an emulsion containing excess cholesterol has stood for a day or two, it is apparent that a considerable portion of the cholesterol has collected in an upper cloudy layer. Examination under the microscope reveals the presence of clumps of droplets coated with cholesterol so finely divided that no crystal structure is visible. A typical clump of this kind is shown in Fig. 5,A. Note the irregular shape of the clump and the white color which

is due to the minute particles of cholesterol in the oil-water interface.

4. For the formation of large cholesterol crystals from the minute particles, it is necessary for the emulsifying film of sodium glycocholate to be broken so that cholesterol can come in contact with the saturated fat droplets. Under such circumstances it would be expected that the minute particles would dissolve and reprecipitate out in larger units. There proved to be two relatively simple methods of breaking the glycocholate film on the droplets: first, add a trace of acid which converts the glycocholate to glycocholic acid which is not an emulsifying agent; or second, allow the sample to dry, thus causing the film of the hydrophilic emulsifying agent to crack.

The effect of allowing the specimen shown in Fig. 5,A to stand in the air for six hours is shown strikingly in Fig. 5,B. Note the disappearance of the minute particles of cholesterol and the formation in their stead of the fan-like, feathery crystals similar in all essential respects to those reproduced in Fig. 1,B. This process is accompanied by the release of the greater portion of the fat solvent. The phenomenon demonstrated in Fig. 5,A and B is shown in an even more striking fashion in Fig. 6, in which (A) represents the original clump and (B) and (C) the same after standing four and twenty hours, respectively. Note the relatively large size of the particles especially in (C) and that they are suspended in a drop of fat the outline of which is clearly visible.

5. It would appear obvious that the formation of a mass of interlocking crystals by the mechanism described in the previous paragraph would yield a relatively firm "stone". This was demonstrated by pouring fat supersaturated with cholesterol in glycocholate solution, emulsifying, allowing to

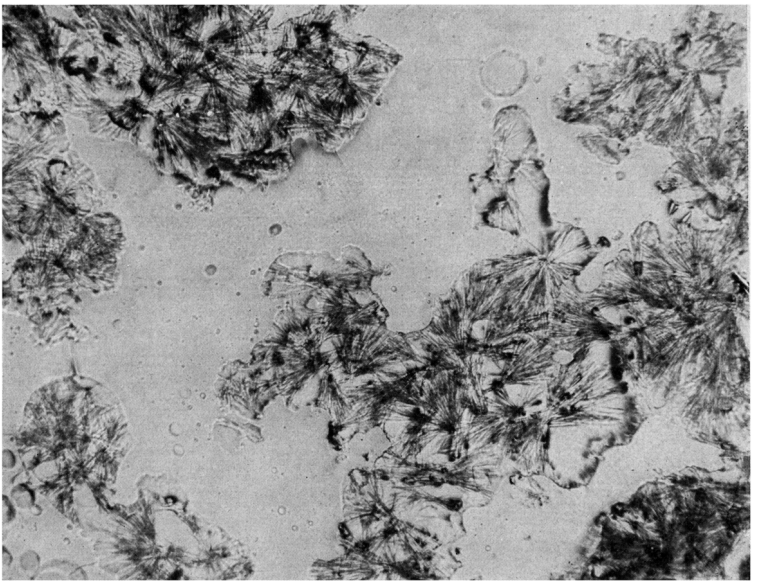


FIGURE 5B. Same as Figure 5A after 6 hours. (x70).

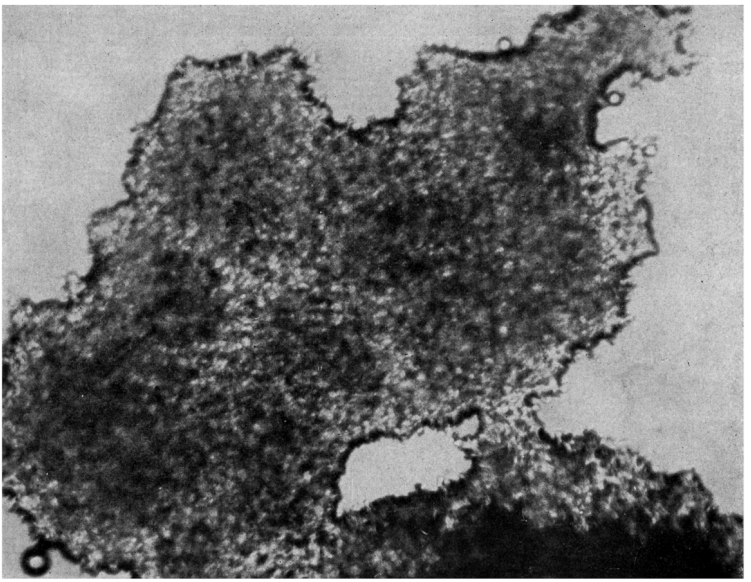


FIGURE 6A. Growth of cholesterol crystals in a cholesterol-fat clump. Original clump. (x70).

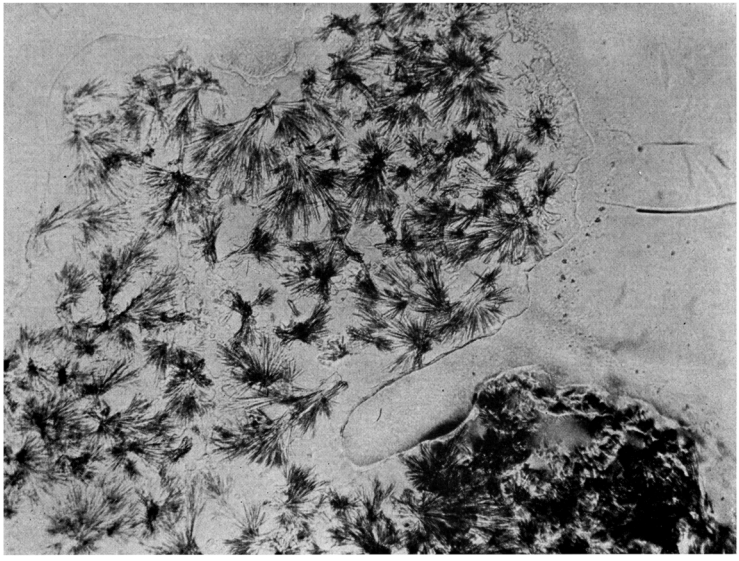


FIGURE 6B. Same as Figure 6A after 4 hours. (x70).

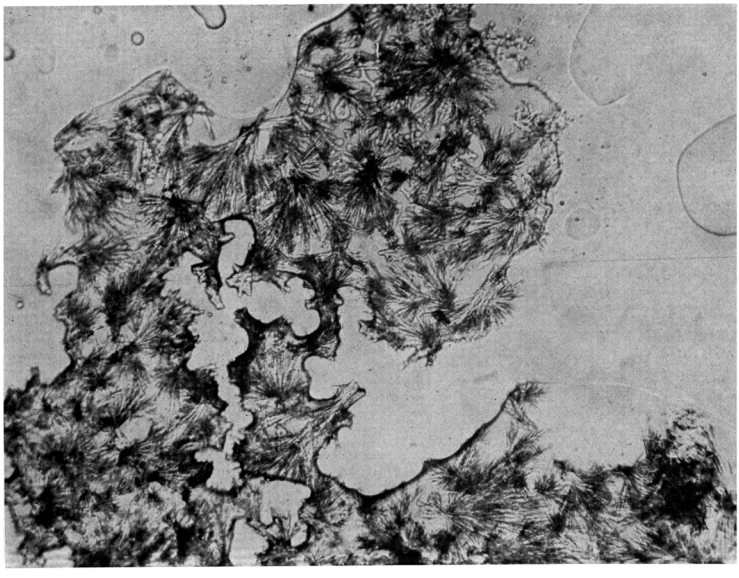


FIGURE 6C. Same as Figure 6A after 20 hours. (x70).

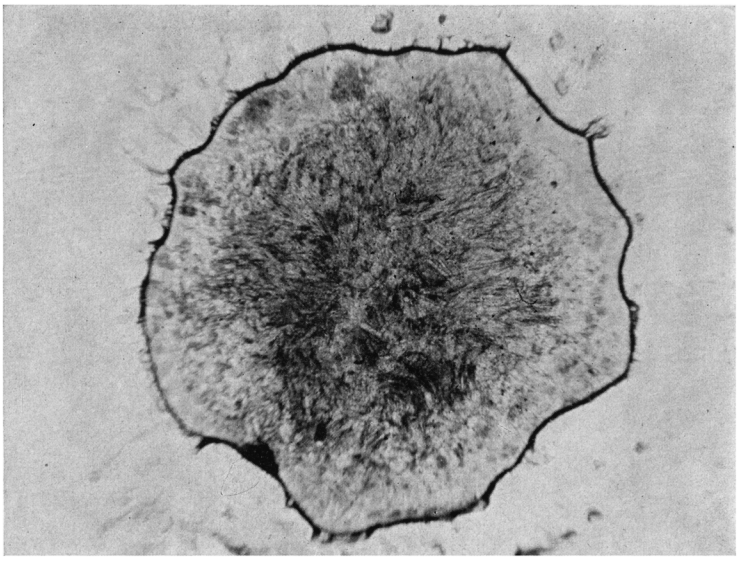


FIGURE 7. Synthetic pure cholesterol stone. (x10).

FIGURE 8. Portion of synthetic pure cholesterol stone. (x70).

stand for some time, and then separating the mass of fat and cholesterol from the remainder of the emulsion by centrifuging. After absorbing the excess fat with blotting paper, it was formed into a ball and dried. The result was a relatively hard crystalline mass, simulating the natural pure cholesterol stone in appearance, composition, and properties. To prepare a photograph, a small ball of the cholesterol mass was flattened out on a microscope slide, dried, and digested at body temperature for four weeks. The result is shown in Fig. 7. Although the magnification is only ten diameters, the radial crystalline structure characteristic of the natural stones is clearly revealed. Fig. 8 is a section of the preparation shown in Fig. 7, magnified seventy times. The higher magnification shows in a striking way how a coherent body is obtained as a result of the laying down of an interlacing mass of needle-like crystals.

In the light of the above series of experiments the mechanism of the formation of cholesterol stones in the absence of inflammation due to infection is believed to be as follows:

In bile stasis resulting from anatomical or physiological abnormalities[1] the bile collects and concentrates in the gall bladder where it may remain for a long period. During this period of stasis there may be an infiltration of cholesterol from a hypercholesterolated blood and a decrease in the amount of the alkali cholates which are responsible for retaining the fat in the form of an emulsion as well as the cholesterol in the dispersed state. In the absence of infection, a decrease in alkali cholates may result from either or both of the following causes: (1) a change in the pH of the bile from the alkaline to the acid side thereby converting the alkali salt to the insoluble glycocholic acid which is neither an emulsifying agent for fat nor a peptizing agent for

[1] Cf. Aschoff: *Lectures on Pathology*, 194 (1924).

cholesterol; or (2) a physiological change in the gall bladder wall which allows resorption of the alkali cholates.

There is no doubt that the first factor above mentioned will contribute to the precipitation of cholesterol since gall bladder bile is normally acid while hepatic duct bile is alkaline. This normal change in pH is probably accentuated in bile stasis.

In the normal gall bladder there is little or no resorption of alkali cholates. Since pure cholesterol stones are found in gall bladders that show no signs of inflammation, past or present, it follows that any resorption of cholates that leads to the formation of a pure cholesterol stone must result from a physiological derangement that does not produce histological changes. Clinical irritation that is not sufficiently severe to leave a permanent change in the tissue may be a contributing factor. Thus a reflux of pancreatic juice into the gall bladder causes cholecystitis and results in marked absorption of cholates.[1] The injection of bacteria produces a similar effect but this changes the whole picture, causing lesions and leading to a different type of stone.

The disappearance of alkali cholate either by conversion to glycocholic acid or by resorption, causes precipitation of cholesterol. This is, of course, most marked in highly hypercholesterolated bile. The excess cholesterol collects around the fat droplets which tend to coalesce as the cholate is gradually removed. Clumps of fat interspersed with cholesterol result and the process of solution of the finely divided particles and subsequent reprecipitation in large needle-like crystals, binds the mass together. The continuation of this process for a long period leads eventually to the concrement which consists of relatively large crystals of

[1] Wolfer: *Surg. Gynecol. and Obstet.*, **53**, 443 (1931); Andrews, Dostal, Goff, and Hrdina: *Ann. Surgery*, **96**, 595 (1932).

cholesterol together with a small amount of enclosed fat.

Particular attention should be called to the importance of fat in the synthesis of pure cholesterol stones. Not only does it serve as a collecting agent which brings together the particles of precipitated cholesterol but its solvent action is responsible for the growth of the interlacing crystals. Since the bile contains no solvent for cholesterol except the fat, the presence of the latter is essential in the formation of pure cholesterol stones such as those shown in Figs. 2 and 7.

### GALLSTONES FORMED WITH INFECTION

The marked inflammation accompanying an infection in the bile duct or gall bladder introduces into the bile irreversibly precipitating hydrophilic colloids such as serum albumin, globulin, and fibrin. If cholesterol or calcium bile pigments separate in the presence of such colloids, there is mutual adsorption with the result that the whole is united into a coherent mass giving what is sometimes termed a colloid-crystalline stone as distinct from the pure crystalline stone considered in the last section. Since approximately two-thirds of the gallstones are of inflammatory origin[1] it follows that colloid-crystalline stones are the type most commonly found in the gall bladder.

As has been pointed out, the formation of pure cholesterol stones is probably preceded by the separation of the cholesterol and its concentration on the fat droplets followed by agglomeration and subsequent formation of an interlacing mass of crystals. A similar mechanism may obtain when inflammation resulting from infection is present but in the latter case such a mechanism is not essential since the colloidal material resulting from the inflammatory process may bind the mass firmly into a concrement. The first

[1] Cf. Aschoff: *Lectures on Pathology*, 217 (1924).

step, namely, the precipitation of cholesterol, is doubtless due chiefly to resorption of the alkali cholates by the inflamed gall bladder wall.[1]

The importance of the colloidal binding material in concrement formation is shown by dissolving out first one constituent and then the other from a colloid-crystalline stone. If the crystalline material is removed with a suitable solvent, a firm coherent skeleton of colloidal matter remains which shows the details of structure of the stone. On the other hand, if the albuminous skeleton is dissolved out by antiformin, there is complete disintegration, nothing remaining but a slimy mass of minute crystals.[2]

Since the relative amounts of the various constituents which make up gallstones of inflammatory origin may vary widely, it is obvious that the composition, appearance, and physical properties of the stone will show almost infinite variation. In a stone in which there is a relatively small amount of hydrophilic colloid, the soft irregular masses consisting largely of cholesterol, undergo solution in fat and recrystallization, ultimately giving a radial structure similar to that in the pure cholesterol stone. Adsorption of the bile pigments or their precipitation as the calcium salts[3] stains the stone to a greater or lesser degree depending on the relative amount of pigment adsorbed or thrown down. If the amount of hydrophilic colloids present in the stone is relatively large, crystal growth is inhibited and the formation of long crystals is prevented. Since the hydrophilic colloids lose water and shrink with time, stones containing a large amount of such colloids may become sufficiently friable

[1] Cf. Andrews, Dostal, Goff, and Hrdina: *Ann. Surgery,* **96,** 619 (1932).

[2] Schade: *Med. Klinik,* No. 15 (1911).

[3] The so-called calcium salt of the bile pigments is probably an adsorption complex of indefinite composition.

that they fall to pieces. In other cases the aging may make radial rifts leading out from the center leaving an irregular hollow space which fills with liquid.

The most common form of gallstones are the colloid-cholesterol-calcium pigment stones that are characterized by the presence of concentric rings varying in color. Such concretions are termed layered stones or "common gallstones". In these stones the kernel and the surrounding ring structure are readily distinguished. The former consists largely of albumin, calcium bilirubin, and cholesterol without the presence of a definite ring structure, while in the latter, there are a number of colored rings in crystals of cholesterol.

Unlike pure cholesterol stones, gallstones of inflammatory origin seldom or never occur singly. In rare instances there may be but two or three such stones but as a rule the number is much larger, a hundred or more, in some cases. Obviously the inflammatory process introduces numerous nuclei on which the mixture of cholesterol and hydrophilic colloids will collect. Because of the pressure of the gall bladder the stones are seldom spherical but are faceted and usually of widely varying shapes.

A pure cholesterol stone formed without symptoms of disease frequently becomes impacted in the neck of the gall bladder or the cystic duct. This can readily lead to a disturbance in the sphincter region, to reflex disturbances of bile secretion and to ascending infections of the bile duct and gall bladder. The infection may result in a loosening of the stone which returns to the inflamed gall bladder and becomes the nucleus about which is deposited a colloid-crystalline mass of hydrophilic colloids, cholesterol, and calcium bilirubin. This gives a so-called combination stone consisting of a nucleus of cholesterol not of inflammatory origin and the shell of the colloid crystalline type resulting from the

inflammation due to infection. The infection following the impaction of a pure cholesterol stone may lead to a large number of colloid-crystalline concretions but there can be but one true combination stone in a given gall bladder.

The cause of the concentric rings in the so-called layered or common gallstones is usually attributed to layering as a result of variation in pathological conditions which produces alternate layers of cholesterol and calcium pigment. Schade[1] attributes the formation of a layered rather than a radiating structure to the effect of pressure which flattens out the crystals of cholesterol. This does not account for the beautiful regular formation of alternate dark and light rings. Moreover, in most stones of this type there is distinct evidence of radiating structure and in many cases this is quite marked as shown in the photographs of stones reproduced in Fig. 9.

The appearance of the concentric rings in the so-called layered stones suggests that they may originate as a result of the rhythmic banding first described by Liesegang as a result of his observations of the formation of rings of silver chromate when silver nitrate is allowed to diffuse into a gelatin jelly containing dichromate. Some examples of this Liesegang phenomenon are shown in Fig. 10,A and B. In Fig. 10,A the solution of dichromate-gelatin was poured on a glass plate and after setting to a jelly it was surrounded by a solution of silver nitrate; and in Fig. 10,B a drop of silver nitrate solution was placed in the center of a circle of dichromate-gelatin jelly.

The possibility that the rings in gallstones are not layers but rhythmic bands is an interesting one that has occurred to a number of people but has been taken seriously by few.

[1]Alexander's *Colloid Chemistry*, **2**, 817 (1927).

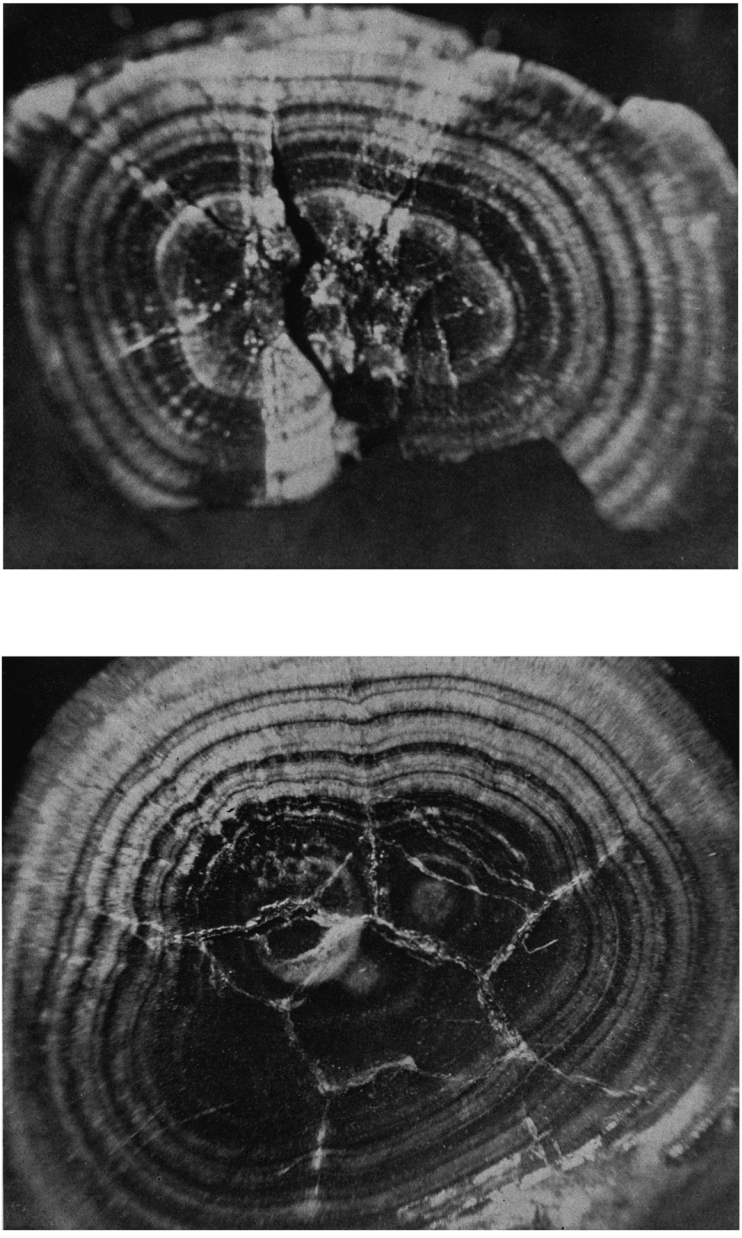
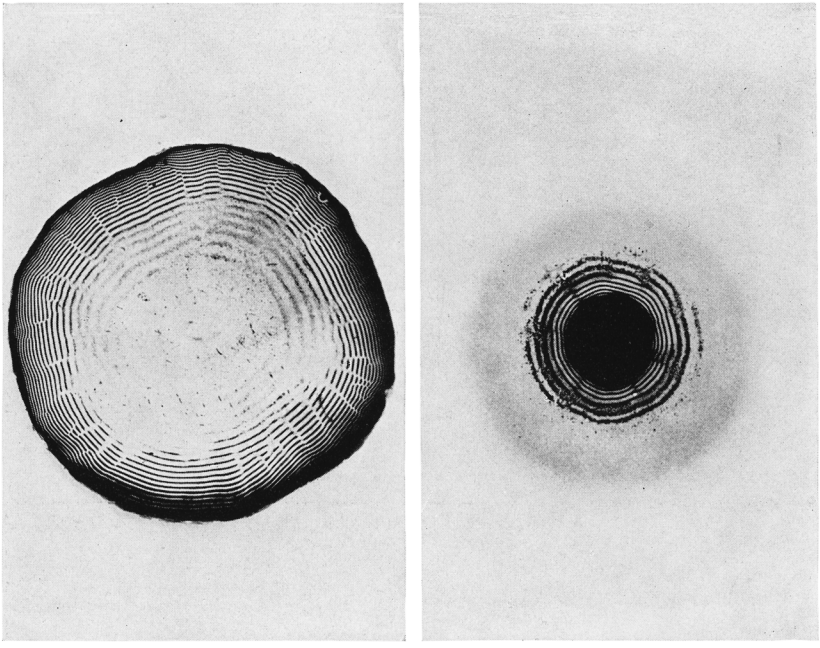


FIGURE 9. Cross-section of gallstones showing both ring and radiating structures. (x10).



A B

FIGURE 10. Rhythmic bands of silver chromate in gelatin formed by diffusion of silver nitrate (A) from the periphery; (B) from the center.

Schade[1] dismisses the suggestion promptly by contending that for the formation of Liesegang rings as a primary process, the diffusion must take place in a jelly which has no points of resistance to diffusion. This, he points out, does not obtain in gallstones owing to the presence of crystalline cholesterol irregularly included in the framework of hydrophilic colloids. Moreover, he rules out the formation of Liesegang rings as a secondary process, since this would give very irregular lines, which do not occur.

Sweet[2] takes the position that the concentric rings are due to the Liesegang phenomenon. He gets around the difficulty confronting Schade by postulating that the cholesterol forms a gel containing calcium into which the bile pigments such as bilirubin can diffuse giving rhythmic bands of calcium bilirubin just as silver nitrate diffuses into dichromate-gelatin jelly and gives rhythmic bands of silver chromate.

A consideration of the gallstone-bile system reveals, however, a marked difference between it and the gelatin-electrolyte system of Liesegang. In the first place, the gallstone is probably not a true jelly at any stage of its history, as assumed by Sweet. And if it did possess a true jelly structure like that of gelatin, rhythmic bands could not form as a result of diffusion since the bile pigments are in colloidal solution and so diffuse but little if at all. Schade's objection to the banding theory on the ground that rhythmic precipitation takes place only in a jelly does not hold, since the phenomenon manifests itself not only by diffusion into jellies but also into relatively non-uniform amorphous and crystalline masses. Accordingly, it is altogether possible that the precipitation of crystalline cholesterol in the presence of hydrophilic colloids will, under certain conditions, give a

[1]Alexander's *Colloid Chemistry*, **2**, 830 (1928).

[2]*Colloid Symposium Monograph*, **8**, 249 (1930).

mass into which the colloidal pigments can diffuse and by interacting with calcium salts carried down with the cholesterol, precipitate colored rhythmic bands. In other words, the very fact that cholesterol is definitely crystalline may be the determining factor in producing rhythmic bands therein, since a mass of small crystals would allow the interdiffusion of the colloidal bile pigments.

If this is a true statement of the case it should be possible to simulate the conditions sufficiently closely that rhythmic bands of calcium bile pigments will be formed in a mass of precipitated cholesterol containing calcium salts. This has actually been accomplished as will be described in the following paragraphs.

1. To show that rhythmic banding will take place in a mass of cholesterol crystals the following experiments were carried out. One gram of gelatin was dissolved in 100 cc of water containing 0.1 gram of potassium chromate and heated to 70°. Into this solution was poured rapidly 25 cc of a hot alcoholic solution of cholesterol containing 2 grams of the pure compound. The cholesterol precipitated immediately in the form of minute crystals. These were matted firmly and uniformly in the bottom of a test tube by centrifuging and the supernatant solution was poured off. The precipitate was then covered with an 8 per cent solution of silver nitrate. Upon standing, the familiar rhythmic bands of silver chromate were formed.

The above procedure was repeated keeping all the factors constant except the amount of gelatin in the solution into which the alcoholic cholesterol was poured. The gelatin solutions contained 0.75, 0.50, 0.25, and 0.05 gram, respectively, in 100 cc. In every case rhythmic bands were formed. With decreasing amounts of gelatin the bands were broader, less distinct, and further apart. When no gelatin was used,

bands were not formed, but crystals of silver chromate were scattered irregularly throughout the mass.

Portions of the precipitate thrown down in the presence of a small amount of gelatin when placed on a watch glass and surrounded with silver nitrate gave rhythmic rings similar to those obtained by diffusion in gelatin.

Since bands of silver chromate in cholesterol are formed in the presence of such minute amounts of gelatin, it is obvious that the rhythmic precipitation is not taking place in a gelatin jelly. The gelatin merely serves to inhibit the growth of the cholesterol crystals and so to give a mass of minute crystals in which the diffusion phenomenon can take place under such conditions that rhythmic bands or rings result.

2. Solutions of bile pigment were prepared in the following way. Twenty-five grams of finely powdered human gallstones were extracted in a Soxhlet tube with 200 cc of ether for two days to remove cholesterol and fat. The residue was dried, washed with hot water, then with 10 per cent acetic acid and finally with water. This residue was dried and ground in small portions with two-normal sodium hydroxide and the filtered solution was used in the experiments. When a portion of the highly colored solution was subjected to dialysis in a cellophane bag, the color did not diffuse, showing that it was in the colloidal state just as it is in the bile fluid. The addition of a dilute solution of calcium nitrate did not result in immediate precipitation of the calcium-pigment complex, but, upon standing, a precipitate settled out which varied in color from reddish brown to olive green, depending upon the degree of oxidation of the pigment.

Although the colloidal bile pigments do not diffuse through gelatinous membranes, it seemed not unlikely, in the light of the above experiments with silver chromate, that a suitably precipitated mass of cholesterol crystals containing

calcium would serve as a medium into which the colloidal pigments would diffuse to form rhythmic bands, thus simulating the process which probably takes place in nature. This hypothesis was confirmed by the following method of procedure. Into 50 cc of a solution containing 0.5 gram of gelatin and 0.5 gram of calcium nitrate at 70° was poured 12.5 cc of a hot alcoholic solution of cholesterol containing 1 gram of the pure compound. After prolonged centrifuging the supernatant solution was removed from the mass of precipitate and portions of the latter were placed on a microscope slide and surrounded by the solution of bile pigments. The specimens were placed in a desiccator containing an ammonia solution to prevent their drying out and to minimize the oxidation of bilirubin to biliverdin. After standing over night rhythmic rings resulted as shown by the photographs reproduced in Figs. 11 and 12. In Fig. 12 the effect of the shape of the mass on the form of the bands is clearly shown. In order to secure a plane surface to photograph, in some cases a cover glass was pressed down gently on top of the specimen flattening out the high places.

A comparison of the synthetic preparations with the natural gallstones reveals a marked similarity in appearance which indicates that rhythmic banding plays a rôle in the formation of the natural stones just as it does in the synthetic ones.

3. The objection may be raised that there is no gelatin in natural gallstones. The answer is that the action of gelatin is not specific. It is only necessary that a protective colloid be present to inhibit the growth of the cholesterol crystals and to serve as a kind of binder. Albumin and fibrin which do occur in natural gallstones may be substituted for gelatin in the synthesis of banded stones. The specimens shown in Fig. 13 were prepared in the same way as those in Figs. 11

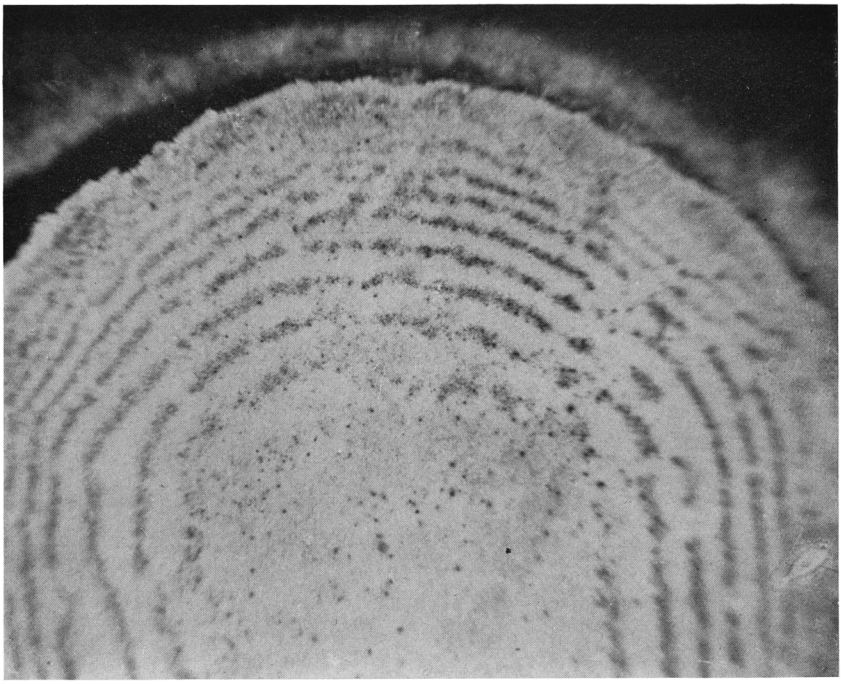


FIGURE 11. Synthetic cholesterol-calcium-pigment gallstone showing rhythmic banding. (x10).

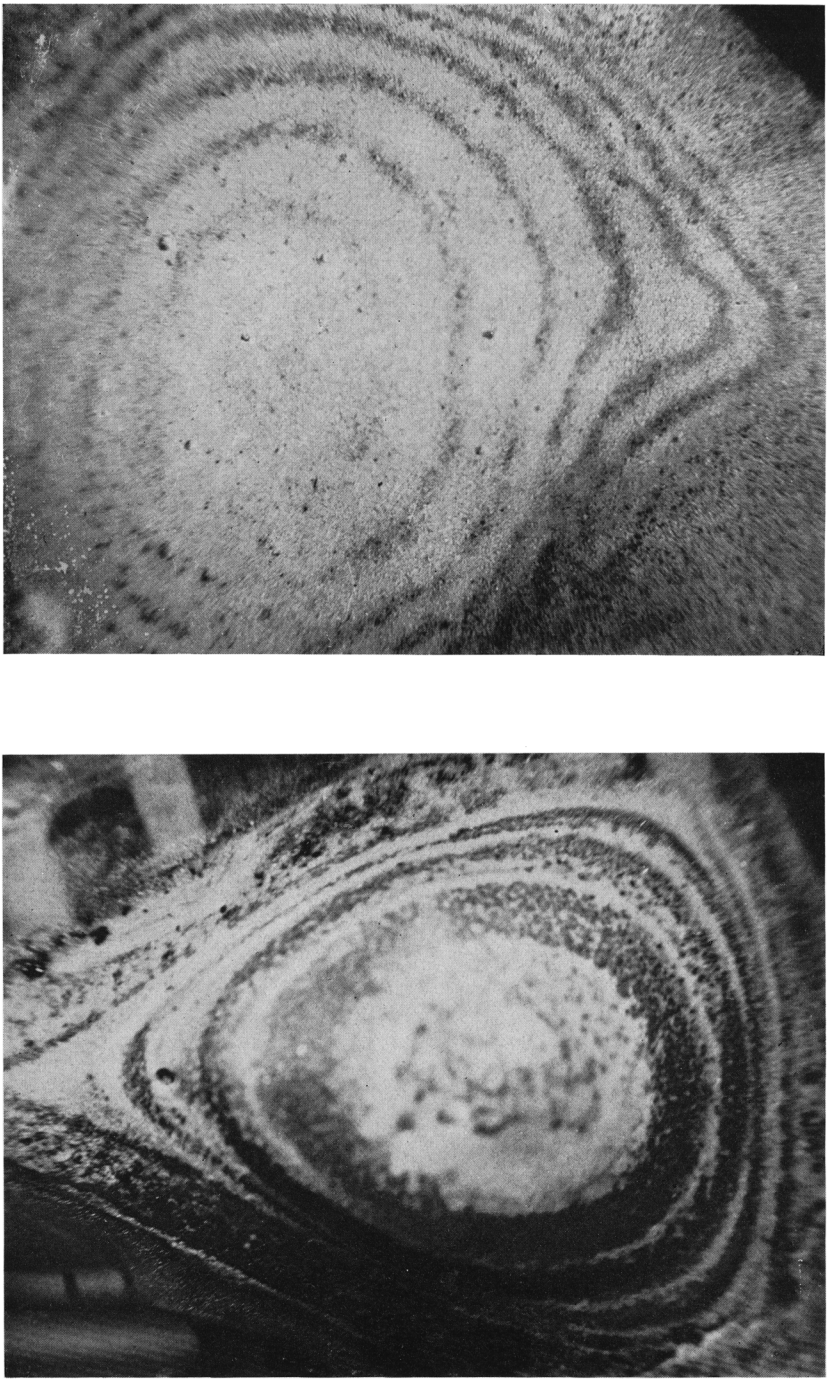


FIGURE 12. Synthetic cholesterol-calcium-pigment gallstones showing how the shape of the mass influences the form of the bands. (x10).

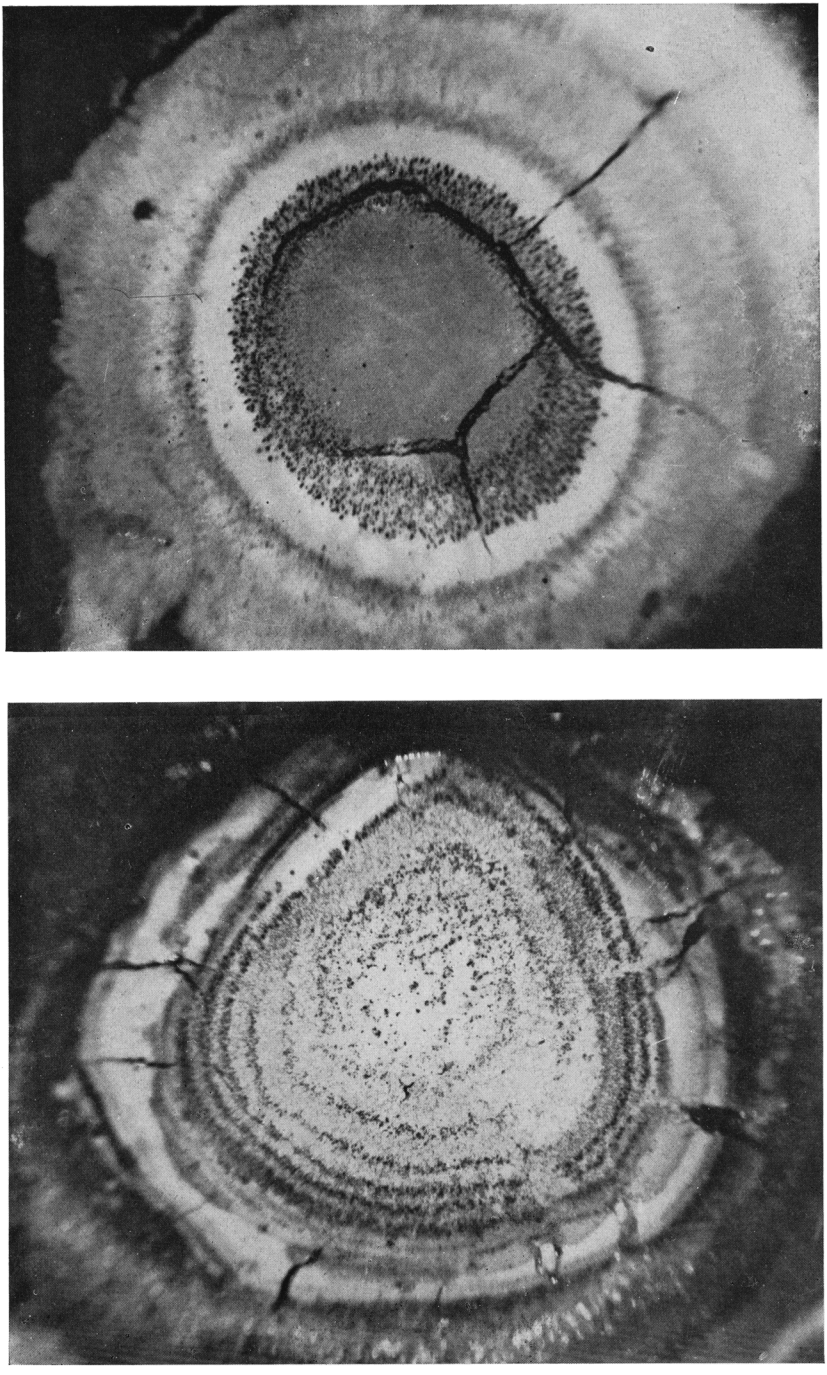


FIGURE 13. Synthetic cholesterol-calcium-pigment stones formed in the presence of a small amount of fibrin. (x10).

and 12, using, instead of gelatin, 50 cc of water in which was peptized 0.1 to 0.2 gram of fibrin.

From a survey of the conditions which result in the formation of the so-called "layered" stones and the experimental results given above, it would appear that the formation of such concretions is favored or initiated by inflammation, which yields irreversibly precipitated protein materials, such as fibrin and albumin. Pathologic changes bring about the precipitation of the cholesterol, carrying calcium with it, the nature of the precipitate depending upon the amount of hydrophilic colloid present. Into this mass the colloidally dispersed bile pigments diffuse and are precipitated in the form of rhythmic bands. The structure and arrangement of the bands is influenced by the shape of the mass, its density due to the pressure of other stones, and by variations in the composition of the bile fluid. After the bands are formed the structure may be invaded by radial crystallization of the cholesterol, cracks may develop, further deposition of the cholesterol may occur, or the stone may undergo alteration in other ways, producing the wide variety of forms which are found in gall bladder disease.

HARRY B. WEISER